Enrichment of Phosphorylated Biomolecules

- Phosphate Affinity Separation using Phos-tag™ MG-bead -

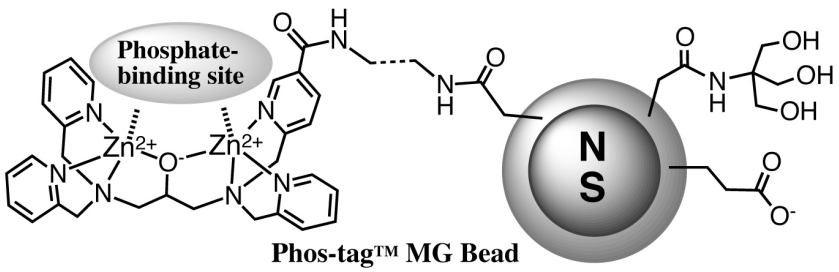
Ver. 2 (2014/7)

1. Introduction

Phosphorylation is a fundamental covalent post-translational modification that regulates the function, localization, and binding specificity of target proteins. Methods for determining the phosphorylation status of proteins (*i.e.,* phosphoproteomics) are thus very important for the evaluation of diverse biological and pathological processes. In 2002, Prof. Koike's group (Hiroshima University) reported that a dinuclear metal complex (*i.e.*, 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex) acts as a selective phosphate-binding tag molecule, Phos-tag™ in an aqueous solution at a neutral pH (*e.g.*, K_d = 25 nM for phenyl phosphate dianion, *Ph*-OPO_3^{2-}). Since then, various methods for phosphoproteome research have been developed using Phos-tag™ derivatives. Here, we introduce a simple and efficient method to enrich phosphorylated biomolecules, such as nucleotides, phosphorylated amino acids, or phosphopeptides. This method is based on magnetic-bead technology using a phosphate-binding tag molecule (a dinuclear zinc(II) complex) linked to a hydrophilic cross-linked agarose coating on a magnetic core particle (Phos-tag™ MG-bead).

2. Description of Phos-tag™ MG-bead

Phos-tag™ MG-Bead (FMN^{2-} binding site ≥1 nmol/μL-beads) provides an efficient procedure for separation of phosphorylated biomolecules including native phosphopeptides from biological samples at physiological pH. The phosphate enrichment procedure needs an appropriate magnet unit and buffers for the binding, washing, and elution processes. The commercially available product (zinc(II)-bound form) is supplied in 2-propanol. Phos-tag™ MG-Bead has no irritant effect on the skin. Stored the beads in a refrigerator at *ca*. 4˚C. Under the condition, the product is stable for at least 6 months.



Phos-tag™ MG Bead

3. Warning and Limitations

Phos-tag™ Mag-Bead is not for use in human diagnostic and the therapeutic procedures. Do not use internally or externally in human or animals. It's used only for research. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water.

4. Advantages of Phos-tag™ MG-bead Method

The operation time for sample collection in the elution fractions is less than 15 min.
The buffers for the binding, washing, and elution processes are all at a physiological pH.
The procedure is almost the same as that for the general magnetic bead method.
Phos-tag™ MG-Bead captures inorganic phosphate ($HOPO_3^{2-}$) and various phosphate dianions ($ROPO_3^{2-}$) bound to nucleotides, phosphorylated sugars, or phosphopeptides.
The ligand (phosphate) exchange rate at the binding site is extremely faster (less than 1 s) than that of other immobilized metal affinity methods using Fe^{3+} or Ga^{3+} complex.

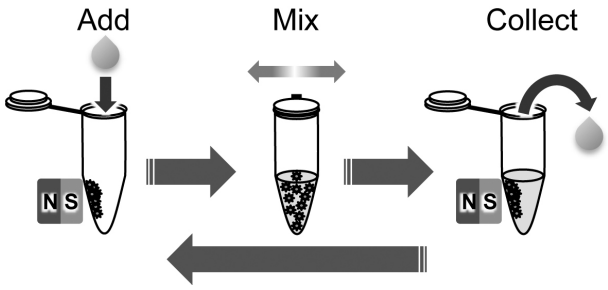
5. Basic Protocol for Separation of Phosphorylated Biomolecules.

1. Phosphate-affinity separation.

1) Phos-tag™ MG-beads (5 ~ 50 µL: Zn^{2+}-bound form) are placed in a 1.5-mL microtube.

2) The beads amassed by using a magnetic stand and the storage solution is removed.

3) The beads are resuspended in 0.10 M Bis-tris–AcOH buffer containing 0.10 M NaCl (pH 6.8, 50 ~ 200 µL) and mixed on a benchtop shaker for 30 s, and then the liquid is removed.
This washing operation is repeated twice.



4) A sample solution consisting of 0.10 M Bis-tris–AcOH buffer (pH 6.8) containing phosphorylated and nonphosphorylated biomolecules (50 ~ 200 µL) is added in the 1.5-mL microtube.
The mixture is incubated with shaking for 3 min at room temperature.

5) The beads are amassed magnetically and the liquid is removed as the flow-through fraction (FT).

6) The beads are resuspended in 0.10 M Bis-tris–AcOH buffer containing 0.10 M NaCl or 0.10 M CH_3COONa (pH 6.8, 200 µL) and slurry is shaken for 30 s. The beads are amassed magnetically and the liquid is isolated. This washing operation is repeated three times and the washings are collected each time as a series of washing fractions, W1 – W3.

7) The beads are resuspended in dist. water (200 µL) and mixed on a benchtop shaker for 30 s, and the liquid is isolated as the final washing fraction (W4).

8) To elute any phosphorylated molecules bound to Phos-tag™ MG-beads, a pyrophosphate buffer (0.10 M $Na_4P_2O_7$–0.10 M AcOH, pH 7.0, 100 µL) is added and mixed for 30 s.
This eluting operation is repeated up to five times to give the respective elution fractions, E1 – E5.
As for phosphopeptide analysis, the sample (E1 fraction) was desalted using a C_{18}-tip prior to mass analysis.

Note: An appropriate additive, such as 0.10 M CH_3COONa or organic solvent, in the washing buffer would produce a good result in the separation of a certain phosphorylated peptide. Please refer to the original article, M. Tsunehiro *et al.*, *J. Chromat. B*, *925*, 86–94, 2013.

2. Reactivation of used Phos-tag™ MG-beads.

1) Used Phos-tag™ MG-beads (*e.g.*, in pyrophosphate-bound zinc form) are treated with 10 times volume of aqueous EDTA·nNa solution (0.10 M, pH 7.0) for 1 h at room temperature.

2) The beads amassed by using a magnetic stand and the liquid is removed.

3) The beads are washed with dist. water (e.g., mixing on a benchtop shaker) and the liquid is removed on a magnetic stand.

4) The beads are resuspended in five times volume of 0.10 M Bis-tris–AcOH buffer containing 10 mM $Zn(AcO)_2$ (pH 6.8) and mixed gently on a benchtop shaker for 30 min, and then the liquid is removed.

5) The beads are resuspended in 0.10 M Bis-tris–AcOH buffer containing 0.10 M NaCl (pH 6.8) and mixed on a benchtop shaker for 1 min, and then the liquid is removed. This washing operation is repeated twice. The beads in active form can be stored in 2-propanol at 4˚C until next use.

6. Solutions for the Phos-tag™ MG-bead Method (at 20˚C)

Sol. A (Sample/Washing buffer): 0.10 M Bis-tris–AcOH (pH 6.8, 100 mL)

# Bis-tris (10 mmol: MW = 209)	2.1 g
# 1.0 mol/L aqueous CH_3COOH (4.0 mmol)	4.0 mL (4.0 g)
# distilled water for preparation of the 100 mL solution	a proper quantity

Note: Check the solution pH of 6.8 ± 0.1 by using a pH meter.

Sol. B (Washing buffer): 0.10 M Bis-tris–AcOH containing 0.10 M NaCl (pH 6.8, 100 mL)

# Bis-tris (10 mmol: MW = 209)	2.1 g
# 1.0 mol/L aqueous CH_3COOH (4.0 mmol)	4.0 mL (4.0 g)
# NaCl (10 mmol: FW = 58)	0.58 g
# distilled water for preparation of the 100 mL solution	a proper quantity

Note: Check the solution pH of 6.8 ± 0.1 by using a pH meter.

Sol. C (Washing buffer): 0.10 M Bis-tris–AcOH containing 0.10 M CH_3COONa (pH 6.8, 100 mL)

# Bis-tris (10 mmol: MW = 209)	2.1 g
# 1.0 mol/L aqueous CH_3COOH (4.6 mmol)	4.6 mL (4.6 g)
# CH_3COONa (10 mmol: FW = 82)	0.82 g
# distilled water for preparation of the 100 mL solution	a proper quantity

Note: Check the solution pH of 6.8 ± 0.1 by using a pH meter.

Sol. D (Reactivation buffer): 0.10 M Bis-tris–AcOH containing 10 mM $(CH_3COO)_2Zn$ (pH 6.8, 100 mL)

# Bis-tris (10 mmol: MW = 209)	2.1 g
# 0.10 mol/L aqueous $Zn(CH_3COO)_2$	10 mL
# 1.0 mol/L aqueous CH_3COOH for pH adjustment at 6.8	a proper quantity
# distilled water for preparation of the 100 mL solution	a proper quantity

Note: Check the solution pH of 6.8 ± 0.1 by using a pH meter.

Sol. E (Elution buffer) 0.10 M $Na_4P_2O_7$–0.10 M AcOH (pH 7.0, 100 mL)

# $Na_4P_2O_7 \cdot 10H_2O$ (10 mmol: FW = 446)	4.46 g
# 1.0 mol/L aqueous CH_3COOH (10 mmol)	10 mL (10 g)
# distilled water for preparation of the 100 mL solution	a proper quantity

Note: Check the solution pH of 7.0 ± 0.1 by using a pH meter.

The following solutions are available as an elution buffer:

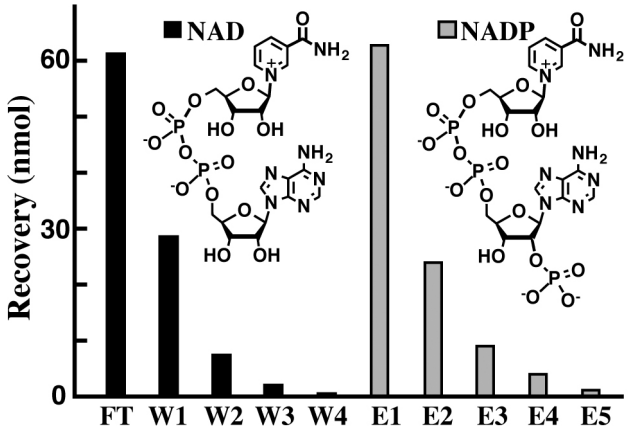
1) 0.10 mol/L EDTA·nNa (pH 7.0, mixing time = 3 min)
2) 3 mol/L aqueous NH_3 (28% NH_{3aq} : H_2O = 1:4, mixing time = 3 min)
3) 0.10 mol/L NaH_2PO_4-NaOH (pH 7.0, mixing time = 1 min).
4) 0.10 mol/L aqueous HCl (mixing time = 1 min)

Note: The Phos-tag™ MG-beads used for a complex sample (*e.g.*, cell lysate) should be discarded.

7. Separation of NAD and its 2'-phosphorylated counterpart NADP

Bead volume: 50 µL (Phos-tag^{TM} MG-bead: FMN-binding site ≈ 3 nmol/µL-gel)
Sample: a mixed solution of 100 nmol NAD and 100 nmol NADP in Sol. A (0.10 mL)
Washing buffer: Sol. A. (0.20 mL x 3) and dist. water (0.20 mL x 1)
Elution buffer: Sol. E (0.10 mL x 5)

The procedure is the same as shown in Section **5-1**. The total time for the phosphate-affinity column chromatography was within 12 min. All the fractions are analyzed by HPLC using a reverse-phase column. The resulting distribution are shown as below.

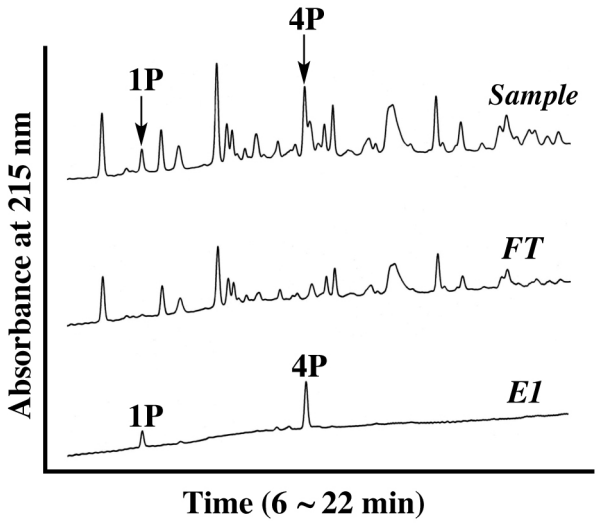


The experimental result shows a distinct separation of NADP from NAD.
The total recovery of the eluted NADP in E1– E5 fractions are more than 99%.

8. Enrichment of Phosphorylated Peptides

Bead volume: 50 µL (Phos-tag^{TM} MG-bead: FMN-binding site ≈ 3 nmol/µL-gel)
Sample: Tryptic digest of 5 nmol β-casein in Sol. A (0.10 mL) :
1P is the monophosphopeptide and 4P is the tetraphosphopeptide.
Washing buffer: Sol. B. (0.20 mL x 3) and dist. water (0.20 mL x 1)
Elution buffer: Sol. E (0.10 mL x 3)

The procedure is the same as shown in Section **5-1**. The total time for the phosphate-affinity column chromatography was within 12 min. All the fractions are analyzed by HPLC using a reverse-phase column. The resulting chromatograms are shown as below.



Both phosphopeptides are efficientyl separated from nonphosphorylated peptides.
The presence of neutral salt NaCl ensures complete elimination of nonphosphorylated peptides.
The recoveries of 1P and 4P in the elution fraction E1 are 69% and 71%, respectively.
The total recoveries of P1 and P4 in the E1 – E3 fractions are more than 95%.
The obtained phosphorylated peptides can be desalted and condensed using a hydrophobic resin such as C18-ZipTip (Milipore).

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